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Stability of the Natural Insecticide Azadirachtin in Aqueous and Organic Solvents

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Abstract: The natural insecticide azadirachtin is most stable in mildly acidic aqueous solutions between pH 4 and 6 at room temperature. It is unstable in mildly alkaline and strongly acidic solutions. It is stable when stored in neutral organic solvents at room temperature for months. While it is relatively stable to heating in the seeds or as a pure solid, it is rapidly destroyed or altered by heating in aqueous solution and methanol. In methanol at 90°C it is quantitatively converted to 3-acetyl-1-tigloylazadirachtinin. © 1998 SCI

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Key words: azadirachtin; nimbin; salannin; *Azadirachta indica*; natural insecticide; stability in solution

1 INTRODUCTION

A tetranortriterpenoid extract of the seeds of the Neem tree (*Azadirachta indica* A. Juss), whose principal constituent is azadirachtin (**I**) (Fig. 1), but including other related triterpenoids such as nimbin (**II**) and salannin (**III**), has been explored over the past 25 years, showing it to be one of the most powerful natural pesticides available to us today.[1–6] Its translation into a commercial product, however, has been slow, partly because of the instability of azadirachtin under various conditions of isolation, storage and use. Some early products offered to the market failed because too little was understood about the stability of the material. A number of products are now available where these problems have been recognized and overcome. While the necessary information required to produce a stable product is evidently available to the processors, little of it has appeared in the scientific literature. The stability of azadirachtin to light in field conditions,[7] and in the laboratory has been explored[8–11] and it would appear that its stability can be improved greatly by addition of UV stabilizers.[10] Its stability in natural and buffered waters has been studied for its survival in agricultural and forestry practice. There was little difference found in the rate of decomposition in buffered distilled water[12] between pH 4 (half-life 19 days at 20°C) and pH 7 (half-life 12·9 days at 20°C), but decomposition was rapid at pH 10. Decomposition was slower in unsterilized pond water (pH 7·36) than in the same water sterilized (pH 8·08).[12] The faster rate in sterilized water can be attributed to the difference in pH, but bacterial decomposition evidently was not affecting the compound. It was concluded that formulation with surfactants considerably retarded decomposition.[12] In a later study the rate of decomposition was constant from pH 4 to pH 6 (half-life 11·6 days to 8·6 days) at 35°C, but increased steadily at higher pH.[13] Stability fell rapidly at higher temperature. At pH 7 the half-life was 11·75 days at 25°C but only 20·5 h at 45°C.[13] By extrapolation from their results we conclude that the half-life of azadirachtin was 24 days at pH 7 and 20°C. Less has been said about its stability in organic solvents. Hull *et al.*[14] report that a methanolic solution of azadirachtin (1·0 mg ml^{-1}) stored at −20°C was stable for at least six months. In early isolation and structure determination work we found that azadirachtin was very sensitive to acid and alkali,[15] and it has been so described more recently.[16] In order to provide more information to those using azadirachtin in experimental or practical conditions, we have summarized a number of experiments we have

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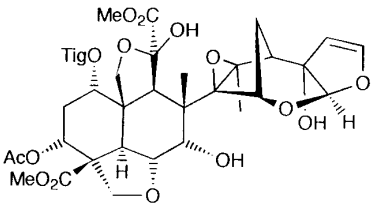
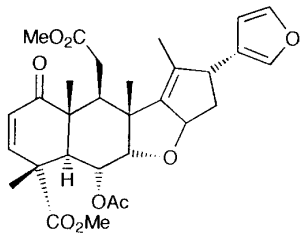
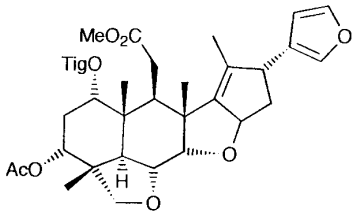


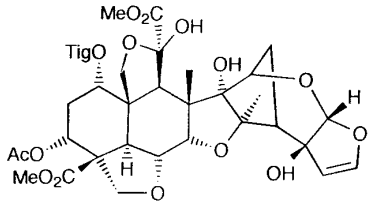
(I) azadirachtin



(II) nimbin



(III) salannin



(IV) 3-acetyl-1-tigloylazadirachtinin

Fig. 1. Structures of compounds discussed.

conducted on the stability of azadirachtin, particularly in solutions.

2 EXPERIMENTAL

2.1 Chemicals

Methanol, acetonitrile and acetone were HPLC grade, toluene was GPR grade, dried over sodium, water was glass-distilled, but not further purified. Carbon dioxide for chromatography was commercial grade (BOC plc). The stock buffer solution was prepared by dissolving potassium orthophosphate (K_3PO_4, 3·893 g), citric acid (6·008 g), boric acid (1·769 g) and diethylbarbituric acid (5·226 g) in water (1 litre). Buffer solutions in the range pH 2 to pH 11 were prepared by adding aqueous sodium hydroxide (0·2 M) to 50 ml aliquots of the stock solution. Measurement of pH was made with an analogue pH meter (Pye Unicam, Cambridge).

Azadirachtin, nimbin and salannin were isolated from seeds of *A. indica* from Sri Lanka as described by Johnson and Morgan.[17] Purity was determined by supercritical fluid chromatography (SFC) and HPLC and by [^{1}H]NMR spectroscopy at 270 MHz.

2.2 Chromatography conditions

High pressure liquid chromatography (HPLC) was carried out as described earlier,[17] using a Spherisorb® ODS analytical column, 5 μm particle size, 250 × 4·6 mm ID (HPLC Technology, Macclesfield) with isocratic acetonitrile + water (1 + 1 by volume) at a flow-rate of 1·0 ml min^{-1}. Detection was by UV absorption at 217 nm, with data handled by a Shimadzu C-R3A integrator (Dyson Instruments, Houghton-le-Spring, England).

SFC was also carried out as described previously,[17,18] using carbon dioxide + methanol (24 + 1 by volume) at 20·6 MPa (3000 psi; 207 bar) and 55°C at a flow rate of 2 ml min^{-1} through a Spherisorb® cyanopropyl silica column (150 × 4·6 mm ID) of 5 μm particle size (HPLC Technology). Detection was by UV absorption at 217 nm, with data handling as above.

2.3 pH stability

Azadirachtin (80 mg) was dissolved in methanol (10 cm^3) and aliquots of this (0·5 cm^3) were diluted with the prepared buffer solutions from pH 2 to pH 11 (Section 2.1) to 10 cm^3, and immediately analysed by HPLC. The solutions were kept in the dark at room temperature and analysed by HPLC at regular intervals appropriate to the rate of decomposition (varying from minutes to days) until decomposition was complete, or up to 60 days. Another standard solution of azadirachtin was prepared by diluting the same stock solution (0·5 cm^3) with methanol (10 cm^3). This was analysed by HPLC, and the integral value of the peak area recorded as 100%. This solution was refrigerated and analysed each day that test solutions were analysed, and the integral values between standard and test compared each time. A new standard solution was prepared each month. Experiments were conducted in duplicate. A plot of decomposition against time was prepared for each pH value, the mean values of half-life, calculated from plots of ln (fraction of material remaining) against time, are given in Table 1. In another experiment solutions in the same pH range were prepared by mixing

TABLE 1
Half-Lives of Azadirachtin at Different pH at Room Temperature in Aqueous Buffer Solution, and with pH Adjusted Initially by Aqueous Sodium Hydroxide or Hydrochloric Acid, and the Fraction Remaining after 60 Days Incubation, as Measured by HPLC with UV Absorption

	Buffered		*Unbuffered*
pH	*Fraction remaining at 60 days* (%)	*Half-life* (*days*)[a]	*Half-life* (*days*)
2	7	15	nd[b]
3	19	24	nd
4	45	49	37
5	45	50	42
6	35	37·5	45
7	4·5	19·4	38
8	0	3·35	4·2
9	—	(7·0 h)	(41·0 h)
10	—	(1·3 h)	(2·3 h)
11	—	(~6 min)	(~6 min)

[a] Mean of two determinations.
[b] nd = not determined.

2 M aqueous sodium hydroxide and 2 M hydrochloric acid to obtain the correct pH value. The solutions were kept in the dark and analysed as for the buffer solutions.

2.4 Heat stability: whole seeds

Two samples of whole *A. indica* seeds (5 g each) were heated at 55°C and 100°C for 24 h. The triterpenoids were then extracted as described earlier.[17] Essentially, the seeds were ground three times with methanol, filtered and the methanol, after partial evaporation and addition of water, was partitioned against dichloromethane. The dichloromethane was evaporated and redissolved to a final volume of 10 cm^3 in a volumetric flask, and this solution used for analysis of the content of nimbin, salannin and azadirachtin by SFC. Results were compared with control samples of unheated seeds from the same lot, extracted in the same way.

2.5 Heat stability: solid compounds

Samples of pure nimbin, salannin and azadirachtin (approx. 12 mg each) were heated in an oven as solids at 55°C and 100°C for 24 h, then 10 mg of each, weighed accurately, were dissolved in methanol in a volumetric flask (10 cm^3) and analysed by SFC. Decomposition was measured by comparing peak areas from the heated samples and those of unheated controls.

2.6 Heat stability in water and methanol

Samples of nimbin, salannin and azadirachtin (10 mg each) were dissolved separately in either methanol (10 cm^3) or water + methanol (95 + 5 by volume, 10 cm^3), and then refluxed for 24 h. After 24 h the methanol solution was cooled and analysed by SFC. The water-methanol solution was evaporated to dryness under vacuum, the residue re-dissolved in dichloromethane (10 cm^3) and also analysed by SFC.

An aliquot of the stock solution of azadirachtin in methanol (Section 2.3, 0·5 cm^3) was diluted with either methanol, water or the buffer solution at pH 7 prepared above (Section 2.3) to a total volume of 10 cm^3. The methanol solution was analysed by SFC and the water and buffer solutions were analysed by HPLC. Peak areas obtained from each initial injection were treated as 100%. Aliquots of these solutions (0·5 cm^3) were placed in screw-capped Reacti-vials (Pierce Europe, Oude Beijerland, Netherlands), and kept at 50°C or 90°C and analysed by HPLC at regular intervals until decomposition was complete.

2.7 Stability on storage in solvents

An aliquot of the stock solution of azadirachtin in methanol (Section 2.3, 0·5 cm^3) was diluted with either methanol, acetone or toluene (10 cm^3 total volume for each solution) and azadirachtin content checked by SFC. These solutions were stored in the dark at room temperature for nine months, analysing them regularly, daily for the first week, then every three days for the next three weeks, and finally once a week, and comparing peak areas with the external standard.

3 RESULTS AND DISCUSSION

3.1 pH stability

From a preliminary experiment we noticed a difference in the rate of decomposition of azadirachtin in sodium hydroxide and alkaline buffer, so two series of experiments were run in parallel, with buffered and unbuffered pH. The half-lives of azadirachtin at pH 2 to pH 11 and room temperature and the amount left at the end of the 60 day experiment are given in Table 1. Some typical experiments are shown in Fig. 2.

3.2 Heat stability

Because the amount of triterpenoids extractable after heating seeds has been found to increase slightly, we cannot give accurate values for the experiment in which nimbin, salannin and azadirachtin were extracted from seeds left for 24 h at 55°C and 100°C. The SFC chromatograms are shown (Fig. 3). While there was no

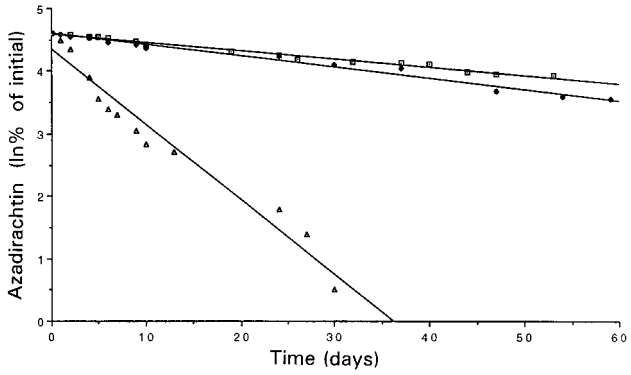


Fig. 2. Decomposition of azadirachtin in aqueous buffer at pH (□) 4, (◆) 6 and (△) 8 at room temperature. Measurement by HPLC with UV absorption at 217 nm.

noticeable decline in either the nimbin or salannin extracted, nor in the azadirachtin in the sample at 55°C, there is an appreciable loss of azadirachtin at 100°C, confirming the results of Ermel *et al.*[19]

When the same three compounds were heated as pure solids, it was possible to give accurate quantifications. Again there no degradation was found at 55°C, but there was some loss, particularly of salannin at 100°C.

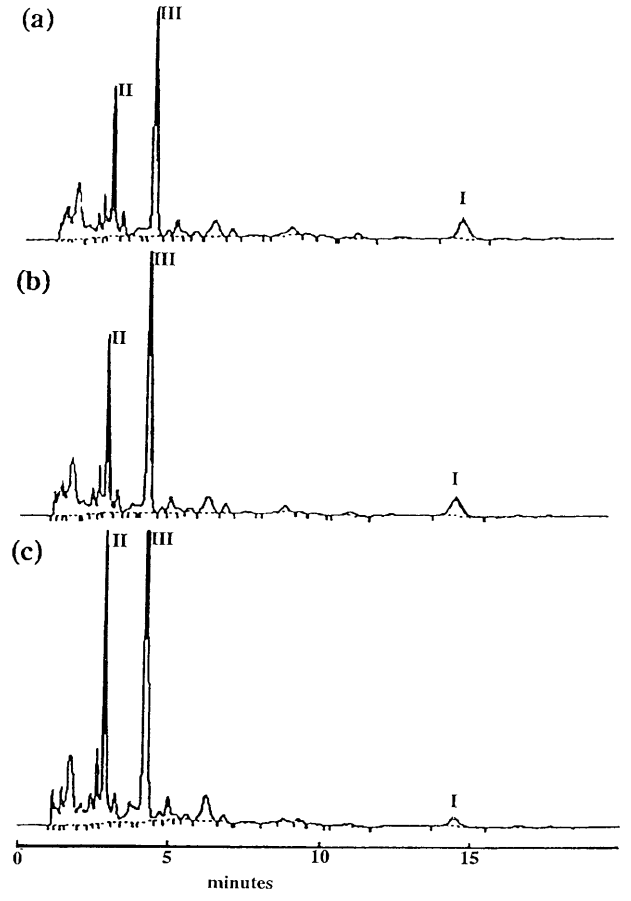


Fig. 3. SFC chromatograms of extracts of Neem seeds (a) control sample of unheated seeds, (b) seeds heated at 55°C and (c) seeds heated at 100°C for 24 h, and then extracted by a standard procedure. Compounds are (**I**) azadirachtin, (**II**) nimbin and (**III**) salannin. Note that the salannin peak is off-scale in (c).

TABLE 2
Decomposition of Pure Nimbin, Salannin and Azadirachtin after Heating to 55°C and 100°C for 24 h as Solids, and in Refluxing Methanol and Water

	Solid degraded (%)		*Degraded in solution* (%)	
Compound	*55°C*	*100°C*	*Refluxing methanol*	*Refluxing water*
Nimbin	0·0	0·0	0·0	0·0
Salannin	0·0	29·3	10·7	33·0
Azadirachtin	0·0	7·6	40·8	100·0

Only 8% of azadirachtin was lost in 24 h at 100°C (Table 2). In solution, nimbin was stable to refluxing for 24 h in water and methanol, but both salannin and azadirachtin were unstable in refluxing methanol (64°C) and even less stable in refluxing water. The product obtained by heating in methanol depended upon the temperature used. At 50°C two unidentified products were formed, in refluxing methanol three product peaks were observed (t_R 11·7, 15·6 and 16·6 min, Fig. 4); the first of these, the major product, was identified by its NMR spectrum and retention time as 3-acetyl-1-tigloylazadirachtinin (Fig. 1; **IV**), a known rearrangement product of azadirachtin.[20,21] The minor products were not isolated. At 90°C azadirachtin was converted quantitatively to (**IV**) in two days. No UV-absorbing products were visible in the chromatograms of azadirachtin heated in aqueous solution.

We then conducted time-course experiments on the decomposition of azadirachtin in methanol and water. In methanol at 50°C it had a half-life of 6·96 days and at 90°C the half-life was 11·7 h. In both cases plots of ln [conc] against time showed that the decomposition was first-order. The regression coefficient was 0·994 for all cases. In distilled water (pH 6·7) at 50°C the half-life was 14·9 h and at 90°C it was 18 min. In phosphate buffer at pH 7, the half-life was 9·9 h at 50°C and 8·9 min at 90°C. From ln[conc] plots it was seen that decomposition was rapid for the first few hours and then settled to a linear first-order decomposition.

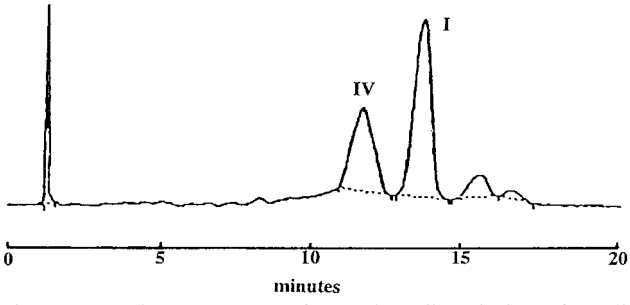


Fig. 4. SFC chromatogram of a methanolic solution of azadirachtin after refluxing for 24 h, (**I**) azadirachtin, (**IV**) 3-acetyl-1-tigloylazadirachtinin.

Azadirachtin solutions in methanol, acetone or toluene stored in the dark at room temperature showed no deterioration during nine months of the experiment.

4 CONCLUSIONS

Our studies have been directed towards a better understanding of the stability of Neem compounds during isolation and in subsequent storage. We included the two compounds nimbin and salannin in the early experiments since these are frequently the triterpenoids present in the seeds in greatest quantity. They perform as markers during isolation. We have shown that nimbin is essentially a very stable substance, salannin less so, but both are more stable than azadirachtin to heating in solution. The presence of nimbin and salannin in an extract when azadirachtin is absent or low suggests that some azadirachtin has been lost in processing.

Initially we used SFC for quantification since that is the fastest method,[17] but as aqueous solutions are not suitable for injection in SFC, in the later experiments, where water or methanol was used routinely, quantification was by reverse-phase HPLC.

Our results on the decomposition of azadirachtin in buffered aqueous solution at room temperature agree very well with those of Szeto and Wan[13] at 35°C that stability is greatest between pH 4 and 5, begins to fall at pH 6 and declines rapidly at pH 7. In unbuffered solution the pH changed during the course of the experiment, so the different half-lives are explained, and the compound is most stable at pH 6 unbuffered. It is evident from our experiments and those quoted[12,13] that azadirachtin is most stable in mildly acidic medium, and solutions should never be permitted to become alkaline. Heating solutions of azadirachtin in water or methanol will lead to some loss of the compound; the higher the temperature, the greater the loss. Aqueous solutions should be avoided for storage of azadirachtin unless they are deep-frozen. Azadirachtin solutions in organic solvents are stable at room temperature, provided they do not (as dichloromethane and chloroform do in sunlight) decompose to give acidic products.

Zanno *et al.*[22] said that azadirachtin was unstable in deuterochloroform solution. That must have been because their deuterochloroform was partially decomposed. We have measured the [^{1}H]NMR spectrum of a solution of pure azadirachtin in deuterochloroform sealed in a tube over a period of two years and found no deterioration in the quality of the spectrum.

Compared to other natural insecticides like pyrethrin and rotenone, azadirachtin is a rather stable substance. Its relative stability, together with its high potency and systemic action[23,24] give high hopes for its future widespread use.

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